Remarks

In view of the above amendments and the following remarks, reconsideration of the outstanding office action is respectfully requested.

Claims 1, 3, 9, and 11 have been amended, claim 2 has been cancelled without prejudice, and new claim 53 has been added. Descriptive support for the claim 1 amendments is found, *inter alia*, in original claim 2, paragraphs [0038], [0039], [0115] with Table 1, [0130], [0132], [0137], [0139] and [0144]. Descriptive support for claim 9 amendments can be found in paragraphs [0042], [0054], and Example 1. Amendments to claim 11 are supported by paragraphs [0043] and [0045]. Descriptive support for new claim 53 is provided in claim 1 and paragraph [0039]. No new matter has been added by way of the above amendments.

Claims 1, 3-6, 9, 11-13, 19-22 and 53 are under examination, and claims 14-18, 23-26, 32-46, and 48-52 stand withdrawn. No excess claim fees are due with this submission.

The objection to claim 11 for informalities is overcome by the above amendments. The objection to claim 11 should be withdrawn.

The rejection of claims 1, 2, 6, 9, 11-13, and 19-22 under 35 U.S.C. § 112, first paragraph, for failing to comply with the written description requirement is respectfully traversed.

The United States Patent and Trademark Office ("PTO") has asserted that the application fails to identify the structure/function relationship of factor VIII proteins and therefore cannot support the genus of factor VIII proteins as claimed. Applicants respectfully disagree, because the specification demonstrates that the structure/function of factor VIII proteins was well known in the art, and factor VIII nucleic acid and amino acid sequences were well known in the art at the time of filing.

As evidence that the structure and function of factor VIII proteins was well known in the art, the specification identifies wild-type factor VIII nucleic acid and amino acid sequences from human, rat, mouse, dog, chimp, and pig by their Genbank accessions. These Genbank accessions are incorporated by reference into the specification. The specification also references the availability of homology and alignment analyses for factor VIII proteins via the HAMSTeRS internet site (see paragraph [0033]). Attached as Exhibit 1 is the referenced

HAMSTeRS alignment of human, porcine, murine, and canine factor VIII, which demonstrates the high degree of homology among these proteins that was known in the art at the time of filing.

With respect to the human sequence, which for the above-noted reasons represents the genus of mammalian factor VIII, the sequences and domains are explicitly identified in paragraphs [0034] – [0038]. The presence of a conserved A1 domain calcium binding site is identified in paragraph [0038] and illustrated in Figure 6, which shows an alignment of corresponding regions of human, murine, porcine, and canine factor VIII.

Moreover, the specification explicitly identifies mutant factor VIII proteins that were previously known in the art and can be modified in accordance with the present invention. These are identified in paragraphs [0041] – [0054], and the contents of cited references are incorporated by reference into the application.

From the foregoing, persons of skill in the art fully appreciated at the time of filing that a known structure/function relationship exists for mammalian factor VIII proteins, whether a wild-type form or a previously known mutant form.

University of California v. Eli Lilly and Co., 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997) is cited by the PTO for the proposition that a general recitation of function is insufficient to define the structure of a protein. This decision confirmed that the single disclosed rat cDNA encoding insulin did not support claims to human cDNA encoding insulin (no species disclosed), mammalian cDNA encoding insulin (1 species disclosed) and vertebrate cDNA encoding insulin (1 species disclosed). However, in sharp contrast to the facts in *University of California*, as noted above many mammalian factor VIII proteins were known in the art prior to the present invention — both wild-type and mutant forms. Importantly, their structural homology was also known (see attached Exhibit 1 and Figure 6 of application). Applicants therefore submit that the recitation and incorporation by reference of six different wild-type mammalian factor VIII proteins and dozens of mutant mammalian factor VIII proteins adequately supports the genus as claimed. The assertion on page 4 of the office action that the specification only supports human factor VIII is therefore improper.

The rejection of claims 9 and 11 is obviated by the above amendments.

For all these reasons, the rejection of claims 1, 2, 6, 9, 11-13, and 19-22 for lack of written description is improper and should be withdrawn.

The rejection of claims 1-6, 9, 11-13, and 19-22 under 35 U.S.C. § 112, second paragraph for indefiniteness is respectfully traversed.

Claims 1 and 6 are rejected for lack of clarity concerning the term "specific activity". Claim 1 has been amended to recite "specific activity, as measured in a one-stage clotting assay". This clotting assay is well known in the art and identified in the specification (see Example 10). The one-stage clotting assay measures the ability of a product—in this case, the claimed product—to shorten the clotting time of hemophilia A plasma in a system containing activated partial thromboplastin. It is called a one-stage assay because, after a preincubation of the activated partial thromboplastin, factor VIII-deficient plasma, and the product being tested to achieve contact activation of plasma, the single step of recalcifying the incubation mixture leads to fibrin clot formation. The one-stage clotting assay is described, and compared to other known *in vitro* assays, in Lundblad et al., "Issues with the Assay of Factor VIII Activity in Plasma and Factor VIII Concentrates," *Thromb. Haemost.* 84:942-948 (2000) (copy attached as Exhibit 2).

Claims 9 and 11 are rejected for lack of clarity in failing to define the term "portion" as it relates to domains A1, A2, A3, C1, and C2. Claim 9 has been amended to instead recite a recombinant factor that is B domainless, which overcomes this basis of rejection. For these reasons, the rejection of claims 1-6, 9, 11-13, and 19-22 for indefiniteness should be withdrawn.

The rejection of claims 1-6, 9, 11-13, and 19-22 under 35 U.S.C. § 102(a) as being anticipated by Wakabayashi et al. "Residues 110-126 in the Factor VIII Heavy Chain Contain a Ca^{2+} Binding Site Required for Cofactor Activity," *Blood, ASH Annual Meeting Abstracts*, 102(11): p542a, Abstract 1988 (2003) ("Wakabayashi") is respectfully traversed.

Wakabayashi is not available as prior art under 35 U.S.C § 102(a). Pursuant to the accompanying Declaration of Philip J. Fay under 37 C.F.R. § 1.132 ("Fay Decl."), Wakabayashi does not evidence knowledge or use of the present invention by others in this country prior to the invention by applicants. Specifically, non-inventors Jan Freas and Qian Zhou prepared and purified reagents under the direction and control of one of the inventors (Fay Decl. ¶4). Neither Jan Freas nor Qian Zhou contributed to the conception of the claimed invention (*Id.*). For these reasons, the rejection of claims 1-6, 9, 11-13, and 19-22 for anticipation by Wakabayashi is improper and should be withdrawn.

The rejection of claims 1, 2, 6, 9, 12, 13, and 19-22 under 35 U.S.C. § 102(b) for anticipation over U.S. Patent No. 5,422,260 to Kaufman et al. ("Kaufman") is respectfully traversed.

The PTO cites Kaufman for disclosing mutant human factor VIII comprising at least one to three amino acid mutations at certain positions and exhibiting procoagulant activity, methods to produce such variants of factor VIII and pharmaceutical compositions of such variants. This rejection is improper, because the PTO has failed to demonstrate that the mutations specified by Kaufman (at amino acid positions 220, 250, 279, 282, 325, 338, 346, 359, 395, 407, 698, 700, 741, 1664, 1680, and 1719) are in or near at least a calcium binding site of the A1 domain. As recited at paragraph [0031], "in or near" means within about five amino acid residues from a residue that directly interacts with Ca^{2+} or Mn^{2+} ions. Also, the PTO has failed to demonstrate that the recombinant factor VIII of Kaufman has a specific activity, as measured by one-stage clotting activity, that is higher than that of a wild-type factor VIII. Given these deficiencies, the rejection of claims 1, 2, 6, 9, 12, 13, and 19-22 for anticipation by Kaufman is improper and should be withdrawn.

The rejection of claims 1, 2, 6, 9, 12, 13, and 19-22 under 35 U.S.C. § 102(e) for anticipation over U.S. Patent Application Publication No. 2005/0100990 to Saenko et al. ("Saenko") is respectfully traversed.

Saenko is cited for teaching methods to increase the procoagulant activity of factor VIII by substituting one or more amino acids in the A2 domain. Saenko further teaches the methods to produce such mutants and pharmaceutical composition comprising such mutant factor VIII. None of these modified residues is present in the A1 domain, and therefore none of these residues involves a calcium binding site of the A1 domain. This rejection is therefore improper. For these reasons, the rejection of claims 1, 2, 6, 9, 12, 13, and 19-22 for anticipation by Saenko should be withdrawn.

The rejection of claim 11 under 35 U.S.C. § 103(a) for obviousness over Saenko in view of U.S. Patent No. 5,859,204 to Lollar et al. ("Lollar") is respectfully traversed.

The teachings and deficiencies of Saenko are noted above. Lollar is cited for teaching a hybrid factor VIII having human and animal factor VIII amino acid sequences, methods of preparation, and use of such hybrid factor VIII. However, PTO has failed to

demonstrate how Loller overcomes the deficiencies of Saenko. For this reason, the rejection of claim 11 for obviousness over the combination of Saenko and Loller is improper and should be withdrawn.

In view of all of the foregoing, applicant submits that this case is in condition for allowance and such allowance is earnestly solicited.

Respectfully submitted,

Date: February 23, 2009

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Exhibit 1: HAMSTeRS Alignment of Human, Porcine, Murine, and Canine Factor VIII

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HUMFVIII    1 ATRRYYLGAVELSWDYMQSD.LGELPVDARFPPRVPKSFPFNTSVVYKKT
PIGFVIII    1 AIRRYYLGAVELSWDYRQSELLRELHVDTRFPATAPGALPLGPSVLYKKT
MURFVIII    1 AIRRYYLGAVELSWNYIQSDLLSVLHTDSRFLPRMSTSFPFNTSIMYKKT
CANFVIII    1 ATRKYYLGAVELSWDYMQSDLLSALHADTSFSSRVPGSLPLTTSVTYRKT

HUMFVIII   50 LFVEFTDHLFNIAKPRPPWMGLLGPTIQAEVYDTVVITLKNMASHPVSLH
PIGFVIII   51 VFVEFTDQLFSVARPRPPWMGLLGPTIQAEVYDTVVVTLKNMASHPVSLH
MURFVIII   51 VFVEYHDQLFNIAKPRPPWMGLLGPTIWTEVHDTVVITLKNMASHPVSLH
CANFVIII   51 VFVEFTDHLFNIAKPRPPWMGLLGPTIQAEVYDTVVIVLKNMASHPVSLH

HUMFVIII  100 AVGVSYWKASEGAEYDDQTSQREKEDDKVFPGGSHTYVWQVLKENGPMAS
PIGFVIII  101 AVGVSFWKSSEGAEYEDHTSQREKEDDKVLPGKSQTYVWQVLKENGPTAS
MURFVIII  101 AVGVSYWKASEGKEYEDQTSQMEKEDDKVFPGESHTYVWQVLKENGPMAS
CANFVIII  101 AVGVSYWKASEGAEYEDQTSQKEKEDDNVIPGESHTYVWQVLKENGPMAS

HUMFVIII  150 DPLCLTYSYLSHVDLVKDLNSGLIGALLVCREGSLAKEKTQTLHKFILLF
PIGFVIII  151 DPPCLTYSYLSHVDLVKDLNSGLIGALLVCREGSLTRERTQNLHEFVLLF
MURFVIII  151 DPPCLTYSYMSHVDLVKDLNSGLIGALLVCKEGSLSKERTQMLYQFVLLF
CANFVIII  151 DPPCLTYSYFSHVDLVKDLNSGLIGALLVCKEGSLAKERTQTLQEFVLLF

HUMFVIII  200 AVFDEGKSWHSETKNSLMQDRDAASARAWPKMHTVNGYVNRSLPGLIGCH
PIGFVIII  201 AVFDEGKSWHSARNDSWTRAMDPAPARAQPAMHTVNGYVNRSLPGLIGCH
MURFVIII  201 AVFDEGKSWHSETNDSYTQSMDSASARDWPKMHTVNGYVNRSLPGLIGCH
CANFVIII  201 AVFDEGKSWHSETNESLTQ......AEAQHELHTINGYVNRSLPGLTVCH

HUMFVIII  250 RKSVYWHVIGMGTTPEVHSIFLEGHTFLVRNHRQASLEISPITFLTAQTL
PIGFVIII  251 KKSVYWHVIGMGTSPEVHSIFLEGHTFLVRHHRQASLEISPLTFLTAQTF
MURFVIII  251 RKSVYWHVIGMGTTPEIHSIFLEGHTFFVRNHRQASLEISPITFLTAQTL
CANFVIII  245 KRSVYWHVIGMGTTPEVHSIFLEGHTFLVRNHRQASLEISPITFLTAQTF

HUMFVIII  300 LMDLGQFLLFCHISSHQHDGMEAYVKVDSCPEEPQLRMK.NNEEAEDYDD
PIGFVIII  301 LMDLGQFLLFCHISSHHHGGMEAHVRVESCAEEPQLRRK.ADEE.EDYDD
MURFVIII  301 LIDLGQFLLFCHISSHKHDGMEAYVKVDSCPEESQWQKKNNNEEMEDYDD
CANFVIII  295 LMDLGQFLLFCHIFSHQHDGMEAYVKVDSCPEEPQLRMK.NNED.KDYDD

HUMFVIII  349 DLTDSEMDVVRFDDDNSPSFIQIRSVAKKHPKTWVHYIAAEEEDWDYAPL
PIGFVIII  349 NLYDSDMDVVRLDGDDVSPFIQIRSVAKKHPKTWVHYISAEEEDWDYAPA
MURFVIII  351 DLY.SEMDNSTLDYD.SSPFIQIRSVAKKYPKTWIHYISAEEEDWDYAPS
CANFVIII  343 ELYDSDMDVVSFDDDSSSPFIQIRSVAKKHPKTWVHYIAAEEEDWDYAPS

HUMFVIII  399 VLAPDDRSYKSQYLNNGPQRIGRKYKKVRFMAYTDETFKTREAIQHESGI
PIGFVIII  399 VPSPSDRSYKSLYLNSGPQRIGRKYKKARFVAYTDGTFKTRKAIPYESGI
MURFVIII  399 VPTEDMSSYKSVYLENGPHRIGRKYKKVRFIAYTDETFKTREFIQHESGL
CANFVIII  393 EPTPNDRSHKNLYLNNGPQRIGKKYKKVRFVAYTDETFKTREAIQYESGI

HUMFVIII  449 LGPLLYGEVGDTLLIIFKNQASRPYNIYPHGITDVRPLYSRRLPKGVKHL
PIGFVIII  449 LGPLLYGEVGDTLLIIFKNKASRPYNIYPHGITDVSALHPGRLLKGWKHL
MURFVIII  449 LGPLLYGEVGDTLLIIFKNQASRPYNIYPHGITDVSPLHSRRLPRGIKHV
CANFVIII  443 LGPLLYGEVGDTLLIIFKNQASRPYNIYPHGINYVTPLHTGRLPKGVKHL

HUMFVIII  499 KDFPILPGEIFKYKWTVTVEDGPTKSDPRCLTRYYSSFVNMERDLASGLI
PIGFVIII  499 KDMPILPGETFKYKWTVTVEDGPTKSDPRCLTRYYSSEINLEKDLASGLI
MURFVIII  499 KDLPIHPGEIFKYKWTVTVEDGPTKSDPRCLTRYYSSFINPERDLASGLI
CANFVIII  493 KDMPILPGEIFKYKWTVTVEDGPTKSDPRCLTRYYSSFINLERDLASGLI

HUMFVIII  549 GPLLICYKESVDQRGNQIMSDKRNVILFSVFDENRSWYLTENIQRFLPNP
PIGFVIII  549 GPLLICYKESVDQRGNQMMSDKRNVILFSVFDENQSWYLAENIQRFLPNP
MURFVIII  549 GPLLICYKESVDQRGNQMMSDKRNVILFSIFDENQSWYITENMQRFLPNA
CANFVIII  543 GPLLICYKESVDQRGNQMMSDKRNVILFSVFDENESWYLTENMQRFLPNA
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HUMFVIII   599  KGVQLEDPEFQASNIMHSINGYVFDSLQLSVCLHEVAYWYILSIGAQTDF
PIGFVIII   599  EGLQPQDPEFQASNIMHSINGYVFDSLQLSVCLHEVAYWYILSVGAQTDF
MURFVIII   599  AKTQPQDPEFQASNIMHSINGYVFDSLELTVCLHEVAYWHILSVGAQTDF
CANFVIII   593  LVVQPHDPEFQLSNIMHSINGYVFDNLQLSVCLHEVAYWYILSVGAQTDF

HUMFVIII   649  LSVFFSGYTFKHKMVYEDTLTLFPFSGETVFMSMENPGLWILGCHNSDFR
PIGFVIII   649  LSVFFSGYTFKHKMVYEDTLTLFPFSGETVFMSMENPGLWVLGCHNSDLR
MURFVIII   649  LSIFFSGYTFKHKMVYEDTLTLFPFSGETVFMSMENPGLWVLGCHNSDFR
CANFVIII   643  LSVFFSGYTFKHKMVYEDTLTLFPFSGETVFMSMENPGLWVLGCHNSDFR

HUMFVIII   699  NRGMTALLKVSSCDKNTGDYYEDSYEDIEAYLLSKNNAIEPRSFSQNSRH
PIGFVIII   699  NRGMTALLKVYSCD[illegible]GDYYDNTYEDIPEFL[illegible]VIEPRSFAQNSRP
MURFVIII   699  FRGMTALLKVSSCD[illegible]YYEDIYEDIPTFL[illegible]VIDPRSFHQNTKH
CANFVIII   693  NRGMTALLKVSSC[illegible]YYEDTYEDIPTFLL[illegible]ENNVIFPRSFSQNSRH

HUMFVIII   749  PSTRQKQFNATTIPENDIEKTDPWFAHRTPMPKIQNVSSSDLLMLLRQS.
PIGFVIII   749  PSASQKQF[illegible]TEPEDDVE.LDPQSGERTQ[illegible]SVFSEDSSMLLGQN.
MURFVIII   749  PNTRFKKF[illegible]TIPENDMEKIEPQFEEIAEML[illegible]QSVSVSDMLMLLGQSH
CANFVIII   743  PSTKEKQLKATTTPENDIEKIDLQSGERTQL[illegible]QSVSSSDLLMLLGQN.

HUMFVIII   798  PTPHGLSLSDLQEAKYETFSDDFSPGAIDSNNSLSEMTHFRPQLHHSGDM
PIGFVIII   797  PAPHGSSSSDLQE[illegible]DDYLPG[illegible]AP[illegible]LRPELHH[illegible]
MURFVIII   799  PTPHGLFLSDEQE[illegible]DDHSPNAI[illegible]GPS[illegible]LRPESHHS[illegible]
CANFVIII   792  PTPRGLFLSDLKE[illegible]DDHSRGAI[illegible]G[illegible]E[illegible]LRPELRHSEDR

HUMFVIII   848  VFTPESGLQLRLNEKLGTTAATELKKLDFKVSSTSNNLISTIPSDNLA
PIGFVIII   845  VLTPEP..............ELKKLDSKMSSSSDL[illegible]PTIPSDFLS
MURFVIII   849  VFTPQP[illegible]LQL[illegible]LTT[illegible]KKLELAVSSLFSNLMT.TTILSDNLK
CANFVIII   840  EFTPEP[illegible]LQLRLNE[illegible]LG[illegible]ELKKLDLKISSSSDSLMTSPTIPSDFLA

HUMFVIII   896  AGTDNTSSLGPPSMPVHYDSQLDTTLFGKKSSPLTESGGPLSLSEENNDS
PIGFVIII   881  AETERTHSLGPP[illegible]F[illegible]QLGRIVLG[illegible]SS[illegible]IGAGVPLSSTE[illegible]
MURFVIII   898  ATFEKTLSSGFP[illegible]P[illegible]KLSTTEFG[illegible]LVGSHVPLKASE[illegible]DS
CANFVIII   890  AATEKTGSLGPP[illegible]VHF[illegible]HLGTIVF[illegible]SHLIQSGVPLELSEE[illegible]DS

HUMFVIII   946  KLLESGLMNSQESSWGKNVSSTESGRLFKGKRAHGPALLTKDNALFKVSI
PIGFVIII   928  ............HESSLGENV[illegible]ESDGI[illegible]AHGPASLTKDDVLFKVNI
MURFVIII   948  NILDSTLMYSQESLPRD[illegible]IENDRL[illegible]HGIALLTKDNTLFKENV
CANFVIII   940  KLLEAFLMNIQESSLRE[illegible]MESNRLF[illegible]GPASLIKDNALFKVNI

HUMFVIII   996  SLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTP
PIGFVIII   968  SLVKT[illegible]TN[illegible]IL[illegible]LL[illegible]NRR.........................
MURFVIII   998  SLMKTNF[illegible]TT[illegible]IENST[illegible]QL[illegible]EV[illegible]
CANFVIII   990  SEVKT[illegible]TTNRKT[illegible]PTLLIENSTSVWQDIMLE[illegible]TEFKEV[illegible]

HUMFVIII  1045  LIHDRMLMDKNATALRLNHMSNKTTSSKNMEMVQQKKEGPIPPDAQNPDM
PIGFVIII  1001  [illegible]T[illegible]DKNTTASGLNHVS..............................
MURFVIII  1047  LI[illegible]T[illegible]TYLRLNHMLN[illegible]TT[illegible]PIP[illegible]
CANFVIII  1040  LIH[illegible]T[illegible]TTALSLNHVSNKTTLSK[illegible]E[illegible]HQKKEDPVP[illegible]ENPDL

HUMFVIII  1095  SFFKMLFLPESARWIQRTHGKNSLNSGQGPSPKQLVSLGPEKSVEGQNFL
PIGFVIII  1018  ..............VI[illegible]KNFLSSEEGPSPELLTSSGSSKSVKGQSSG
MURFVIII  1097  SFSKMLFL[illegible]KKY[illegible]NNSLRSEQEHSPKQLVHLHFFKYVKNQSFL
CANFVIII  1090  [illegible]SKIFFLP[illegible]THGKNSLSSEQFPSPKQLTSLGSEKSVKLQNFL

HUMFVIII  1145  SEKNKVVVGKGEFTKDVGLKEMVFPSSRNLFLTNLDNLHENNTHNQEKKI
PIGFVIII  1055  AERERVMVEEEELSKE...KE[illegible]P[illegible]FLTNSADVRGNDTHSQEKKS
MURFVIII  1147  SEKNKVTVEQDEFTKNIGLK[illegible]P[illegible]SIFLTTLSNVHENRRHNQEKNI
CANFVIII  1136  SE.EKVVVEEDEFTKDTE[illegible]IFP[illegible]SIFRRNLANVEENDTYNQEKKS
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HUMFVIII 1195 QEEIEKKETLIQENVVLPQIHTVTGTKNFMKNLFLLSTRQNVE[illegible]
PIGFVIII 1102 FEEMERREKLVQEKVELPQVYTATGTKNFLRNIFHRSTEPSVEG[illegible]
MURFVIII 1197 QEEIE.KEELIEEKVVLPQVHEATGSKNFLKDILILSTRQN[illegible]
CANFVIII 1184 FEEIERKEKLTQEKVELPQSHTMIGTKNFLKNLFLLSTKQN[illegible]

HUMFVIII 1245 APVLQDFRSLNDSTN[illegible]TAHFS[illegible]LEGLGNQTKQ[illegible]VE[illegible]
PIGFVIII 1152 APVPQDSRSLND[illegible]IAHF[illegible]E[illegible]LEAPGNRT[illegible]
MURFVIII 1244 TPVLQNITSINNST[illegible]IHMEHF[illegible]GLENKT[illegible]
CANFVIII 1234 EPILQDTRSLND[illegible]IHMAKFSKI[illegible]EANLEGLGNQTKQMVE[illegible]
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HUMFVIII 1293 NSTRISSNSPSISSDNVTQRSKRALKQFRLPLEETELEKRIIVDDTSTQWSK
PIGFVIII 1192 --------PSSKSSAPSGSWKDSLKQERLPLEEIRSSRSVVLDSSTSWS.
MURFVIII 1293 --------PSQKNISTQRSKRALEQFRL-----------------STQWLK
CANFVIII 1282 NTTRMSSSSKSQH.VITQRSKRALKQERLNLEEIIHTERKVISNDTSTQWSK

HUMFVIII 1343 NMKHLTPSTLTQIDYNEKEKGAITQSPLSDCLTRSHSIPQANRSPLPIAK
PIGFVIII 1234 --------------------------------------------------
MURFVIII 1319 NSNSLTVSQILSQIL.....TISRSLSN...........Q..........
CANFVIII 1331 NMSHLTQSTLTQIEYNEKEKRAITQSPLSDCSNRSHSIPQANRSSLPVAK

HUMFVIII 1393 VSSFPSIRPIYLTRVLFQDNSSHLPAASYRKKDSGVQESSHFLQGA
PIGFVIII 1234 ----------------------------------------SSPHLQGA
MURFVIII 1368 TSAFP---PID...PFQ.......H...AS...........QESNHFLKET
CANFVIII 1381 TSAEPSVRSSDLTKIPEQSNSSHLPASAFRKKDSGVQEESHFLQEA

HUMFVIII 1439 KKNNLSLAILTLEMTGDQREVGSLGTSATNSVTYKKVENTVLPKPDLPKT
PIGFVIII 1243 KRNNLSLAILTLEMTGDQGKISALGKSAAGPLASGKLEKAVLSSAGLSEA
MURFVIII 1415 KTNNFSLAILTLEMTGDQGKFTSPGKSSTNSVTYKKRENIILKPSLPEE
CANFVIII 1431 KRNNLSLAILTLEMTGDQGKFSSLGKSATNQPMYKKLENTVLLKPGLSET

HUMFVIII 1489 SGKVELLPKVHIYQKDLFPTETSNGSPGHLDLVEGSLLQGTEGAIKWNEA
PIGFVIII 1293 SGKAEFLPKVHIYQKDLLPQKTSNVSHAHGDLEQEIFLQKTRGPVNLNKV
MURFVIII 1465 SGKIELLPKVHIYQKEILPTETSHGSPGHLNLMKEVFLQKIQGPTKWNKA
CANFVIII 1481 SEKVELLPKVHIYQEDSFPTKTSNISPGHLDLMQPIFLQKTQGPVKMNKT

HUMFVIII 1539 NRPGKVPFLRVATESSAKTPSKLLDPLAWDNHYGTQIPKEEWKSQEKSPE
PIGFVIII 1343 NRPG-----------------TPSKLLG--------------WESLEKSPK
MURFVIII 1515 ERHGKVPFLRVATESSAKTPSKLLDPLAWDNHYGTQIPKEEWKSKEKSPE
CANFVIII 1531 NSPGKVPFLKVATESSAKTPSKLLDPLAWDNHYGTQIPEEEWKSQEKSQT

HUMFVIII 1589 KTAFKKKDTI.LSLNACESNHAIAAINEGQNKPEIEVTWAKQGRTERLCS
PIGFVIII 1370 STALRTKDIISLPLDRHESNHSIAAKNEGQAETQREAAWTKQGGPGRLCA
MURFVIII 1563 IISIKAEDTI.LSLDACESNHSIGAANEEQNWPQRETTWVKQGQTKRTCS
CANFVIII 1581 NTAFKRKDTI.LPLDACESSTAAINEGQDKPQREAAWAKQGEPGRLCS

HUMFVIII 1638 QNPPVLKRHQREITRTTLQSDQEEIDYDDTISVEMKKEDFDIYLEDENQS
PIGFVIII 1420 PPPPVLRRHQRDISLPTFQPEEDKMDYDDIFSTETKGEDFDIYGEDENQD
MURFVIII 1611 QIPPVLKRHQREISLPTFQPEEDKMDYDDIFSTEIKGEDFDIYSEDIKQG
CANFVIII 1630 QNPPVEKHHQREITRTTLQSDQEEIDYDDTISIEMKKEDFDIYGDWENQG

HUMFVIII 1688 PRSFQKKTRHYFIAAVERLWDYGMSSSPHVLRNRAQSGSVPQFKKVVFQE
PIGFVIII 1470 PRSFQKRTRHYFIAAVEQLWDYGMSESPRALRNRAQNGEVPRFKKVVFRE
MURFVIII 1658 PRSFQMKTRHYFIAAVERLWDYGMSTS.HVLRNRYQSDNVPQFKKVVFQE
CANFVIII 1680 LRSFQKKTRHYFIAAVERLWDYGMSRSPHILRNRAQSGDVRQFKKVVFQE

HUMFVIII 1738 FTDGSFTQPLYRGELNEHLGLLGPYIRAEVEDNIMVTFRNQASRPYSFYS
PIGFVIII 1520 FADGSFTQPSYRGELNKHLGLLGPYIRAEVEDNIMVTFKNQASRPYSFYS
MURFVIII 1707 FTDGSFSQPLYRGELNEHLGLLGPYIRAEVEDNIMVTFKNQASRPYSFYS
CANFVIII 1730 FTDGSFTQPLYRGELNEHLGLLGPYIRAEVEDNIVVTFKNQASRPYSFYS

HUMFVIII 1788 SLISYEEDQRQGAEPRKNFVKPNETKTYFWKVQHHMAPTKDEFDCKAWAY
PIGFVIII 1570 SLISYPDDQEQGAEPRHNFVQPNETRTYFWKVQHHMAPTEDEFDCKAWAY
MURFVIII 1757 SLISYPEDQ.EGEEPRRNFVKPNETKIYFWKVQHHMAPTEDEFDCKAWAY
CANFVIII 1780 SLISYDEDEGQGAEPRRKFVNPNETKIYFWKVQHHMAPTKDEFDCKAWAY

HUMFVIII 1838 FSDVDLEKDVHSGLIGPLLVCHTNTLNPAHGRQVTVQEFALFFTIFDETK
PIGFVIII 1620 FSDVDLEKDVHSGLIGPLLICRANTLNAAHGRQVTVQEFALFFTIFDETK
MURFVIII 1806 FSDVDLERDMHSGLIGPLLICHANTLNPAHGRQVSVQEFALLFTIFDETK
CANFVIII 1830 FSDVDLEKDVHSGLIGPLLICRSNTLNPAHGRQVTVQEFALVFTIFDETK
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HUMFVIII 1888 SWYFTENMERNCRAPCNIQMEDPTFKENYRFHAINGYIMDTLPGLVMAQD
PIGFVIII 1670 SWYFTENVERNCRAPCHLQMEDPTLKENYRFHAINGYVMDTLPGLVMAQN
MURFVIII 1856 SWYFTENVKRNCKTPCNFQMEDPTLKENYRFHAINGYVMDTLPGLVMAQD
CANFVIII 1880 SWYFTENLERNCRAPCNVQLEDPTLKENFRFHAINGYVKDTLPGLVMAQD

HUMFVIII 1938 QRIRWYLLSMGSNENIHSIHFSGHVFTVRKKEEYKMALYNLYPGVFETVE
PIGFVIII 1720 QRIRWYLLSMGSNENIHSIHFSGHVFSVRKKEEYKMAVYNLYPGVFETVE
MURFVIII 1906 QRIRWYLLSMGNNENIQSIHFSGHVFTVRKKEEYKMAVYNLYPGVFETLE
CANFVIII 1930 QKVRWYLLSMGSNENIHSIHFSGHVFTVRKKEEYKMAVYNLYPGVFETVE

HUMFVIII 1988 MLPSKAGIWRVECLIGEHLHAGMSTLFLVYSNKCQTPLGMASGHIRDFQI
PIGFVIII 1770 MLPSKVGIWRIECLIGEHLQAGMSTTFLVYSKECQAPLGMASGRIRDFQI
MURFVIII 1956 MIPSRAGIWRVECLIGEHLQAGMSTLFLVYSKQCQIPLGMASGSIRDFQI
CANFVIII 1980 MLPSQVGIWRIECLIGEHLQAGMSTLFLVYSKKCQTPLGMASGHIRDFQI

HUMFVIII 2038 TASGQYGQWAPKLARLHYSGSINAWSTKEPFSWIKVDLLAPMIIHGIKTQ
PIGFVIII 1820 TASGQYGQWAPKLARLHYSGSINAWSTKDPHSWIKVDLLAPMIIHGIMTQ
MURFVIII 2006 TASGHYGQWAPNLARLHYSGSINAWSTKEPFSWIKVDLLAPMIVHGIKTQ
CANFVIII 2030 TASGQYGQWAPKLARLHYSGSINAWSTKDPFSWIKVDLLAPMIIHGIMTQ

HUMFVIII 2088 GARQKFSSLYISQFIIMYSLDGKKWQTYRGNSTGTLMVFFGNVDSSGIKH
PIGFVIII 1870 GARQKFSSLYISQFIIMYSLDGRNWQSYRGNSTGTLMVFFGNVDASGIKH
MURFVIII 2056 GARQKFSSLYISQFIIMYSLDGKKWLSYQGNSTGTLMVFFGNVDSSGIKH
CANFVIII 2080 GARQKFSSLYVSQFIIMYSLDGRKWHSYRGNSTGTLMVFFGNVDSSGIKH

HUMFVIII 2138 NIFNPPIIARYIRLHPTHYSIRSTLRMELMGCDLNSCSMPLGMESKAISD
PIGFVIII 1920 NIFNPPIVARYIRLHPTHYSIRSTLRMELMGCDLNSCSMPLGMQNKAISD
MURFVIII 2106 NSFNPPIIARYIRLHPTHSSIRSTLRMELMGCDLNSCSIPLGMESKVISD
CANFVIII 2130 NIFNPPIIAQYIRLHPTHYSIRSTLRMELLGCDFNSCSMPLGMESKAISD

HUMFVIII 2188 AQITASSYFTNMFATWSPSKARLHLQGRSNAWRPQVNNPKEWLQVDFQKT
PIGFVIII 1970 SQITASSHLSNIFATWSPSQARLHLQGRTNAWRPRVSSAEEWLQVDLQKT
MURFVIII 2156 TQITASSYFTNMFATWSPSQARLHLQGRTNAWRPQVNDPKQWLQVDLQKT
CANFVIII 2180 AQITASSYLSSMLATWSPSQARLHLQGRTNAWRPQANNPKEWLQVDFRKT

HUMFVIII 2238 MKVTGVTTQGVKSLLTSMYVKEFLISSSQDGHQWTLFFQNGKVKVFQGNQ
PIGFVIII 2020 VKVTGITTQGVKSLLSSMYVKEFLVSSSQDGRRWTLFLQDGHTKVFQGNQ
MURFVIII 2206 MKVTGIITQGVKSLFTSMFVKEFLISSSQDGHHWTQILYNGKVKVFQGNQ
CANFVIII 2230 MKVTGITTQGVKSLLISMYVKEFLISSSQDGHNWTLFLQNGKVKVFQGNR

HUMFVIII 2288 DSFTPVVNSLDPPLLTRYLRIHPQSWVHQIALRMEVLGCEAQDLY
PIGFVIII 2070 DSSTPVVNALDPPLFTRYLRIHPTSWAQHIALRLEVLGCEAQDLY
MURFVIII 2256 DSSTPMMNSLDPPLLTRYLRIHPQIWEHQIALRLEILGCEAQQQY
CANFVIII 2280 DSSTPVRNRLEPPLVARYVRLHPQSWAHHIALRLEVLGCDTQQPA
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Exhibit 2: Lundblad et al., *Thromb. Haemost.* 84:942-948 (2000)

Review Article

Issues with the Assay of Factor VIII Activity in Plasma and Factor VIII Concentrates

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Key words

Factor VIII, factor VIII assay/concentrates, chromogenic assays

Summary

A review of the literature suggests that assays accurate for the determination of factor VIII in plasma samples may not necessarily retain this accuracy when used for the determination of factor VIII in high-purity factor VIII concentrates such as Hemofil® M. Review of assay data suggests that it is imperative to obtain maximal activation of the factor VIII in the sample with thrombin when using an assay system of isolated coagulation factors such as the two-stage assay or the various chromogenic substrate assays. Based on a combination of ease and reproducibility of performance and correlation of in vivo and in vitro measurements, it is recommended that the one-stage activated partial thromboplastin time performed with plasma from an individual with severe hemophilia A be used for the measurement of factor VIII potency. Chromogenic substrate assays can be used if care is taken to assure optimal activation of factor VIII by thrombin in the assay and the presence of sufficient factor IXa, phospholipid and calcium ions to stabilize factor VIIIa during the assay process.

Introduction

The accurate assay of factor VIII is critical in the management of hemophilia A and will be of increasing importance as new modes of therapy such as continuous infusion and other prophylactic approaches are developed (1-5). A desirable criterion for such an assay is that it should be equally sensitive and accurate with therapeutic concentrates and patient samples after infusion. It is equally important for manufacturers of therapeutic concentrates to have a robust and accurate assay for use in the assignment of potency. The World Health Organization (WHO) and the International Society on Thrombosis and Haemostasis (ISTH) have long been involved in calibrating and distributing standards for use in these assays (6, 7). It has been understood that a concentrate standard (e.g. MEGA I) must be used when assigning concentrate potency (8, 9) and that a plasma standard be used when calibrating clinical assays although there can be issues with this approach (10). It is important, however, that concentrate and plasma assay correlate well in order to make sense out of the clinical results.

The activated partial thromboplastin time (aPTT) (11, 12) has been used extensively for the factor VIII assay both in therapeutic concentrates and in hemophilia A patients. There has been concern that the aPTT does not provide an accurate measurement of the potency of highly purified factor VIII (13, 14). It has been suggested that differences between values obtained with the aPTT and a chromogenic or two-stage assay reflects the presence of activated factor VIII in these preparations (15-17). Another emerging complication for factor VIII assays is the European requirement for the use of chromogenic substrate assay systems for factor VIII (18). The European Pharmacopeia describes a generic chromogenic substrate assay and gives recommendations on the level of purity of the factors used to formulate the assays (generally around 50%) and the concentration of the various components. The method recommends that the reaction to activate the factor X be terminated before all the factor X is activated. The package inserts of the various commercial chromogenic substrate assays do not always contain enough information to know if they meet the criteria in the Pharmacopeia.

The purpose of this review is to critically examine the various assay systems currently available for the assay of factor VIII activity both in therapeutic concentrates and plasma samples. The review also considers the advances in the understanding of the biochemistry of factor VIII that are relevant to the various assay systems. The reader is referred to several recent reviews of factor VIII for general information (19-24).

Mechanism of Action of Factor VIII in Blood Coagulation

A major problem in the assay of factor VIII is that this protein can only be measured in an indirect manner. Factor VIII is not an enzyme but rather a procofactor in the activation of factor X by factor IXa (19-24). Thus, all currently available factor VIII assays measure factor VIII indirectly through its cofactor activity (available only after peptide bond cleavage) to generate factor Xa. Factor Xa is measured

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either in a direct manner such as described below for the chromogenic substrate assay systems or in an indirect manner through the formation of fibrin by thrombin formed by the coagulation cascade.

Activation State of Factor VIII

The assay of factor VIII would be less complicated if factor VIII did not require activation prior to function as a cofactor in the activation of factor X (25, 26). Activation of factor VIII by thrombin results in an observed 30-50 fold (27, 28) or greater (29) increase in cofactor activity. However, the full extent of *potential* factor VIII activation is not known since the activity of single-chain factor VIII, unlike that of the homologous molecule, factor V (30), has not been measured. In the absence of specific data to the contrary, it is generally assumed that single chain factor VIII does not have any cofactor activity (31, 32). However, estimates of baseline activity are flawed also because as with factor VIII, all assays are indirect. Understanding of the activation process is further complicated because species of factor VIII which have undergone some, but not all of the peptide bond cleavages required for full activation, demonstrate enhanced cofactor activity corresponding to the degree of processing (33). For example, it was determined that cleavage of the 80 KD light chain to the 70 KD $A_3C_1C_2$ fragment is associated with a six-fold increase in activity (33). Cleavage of the heavy chain at residues 372-373 yields the heterotrimer, A_1, A_2, $A_3C_1C_2$ (34, 35) and resulted in additional activation but the product was quite unstable. Recent studies (36, 37) with abnormal factor VIII proteins isolated from patients with mild hemophilia A have provided additional evidence for the importance of light chain cleavage at 1689-1690 during the activation process. These studies demonstrated that abnormal factor VIII proteins cleaved poorly at residues 372-373, are still partially activated by thrombin via light chain cleavage. Previously, this cleavage was considered to be not as critical as heavy chain cleavage for the activation process (38, 39); the change in perspective occurred when it became appreciated that light chain cleavage is required for von Willebrand Factor (vWF) dissociation and vWF dissociation from factor VIII is required for cofactor activity (39). The importance of light chain cleavage in the factor VIII activation process has been further demonstrated by Regan and Fay (40) in experiments where the isolated thrombin-cleaved light chain was allowed to associate with the isolated heavy chain. The activity of this hybrid protein was some three-fold greater than the factor VIII obtained from the association of intact light chain and heavy chain.

Proteases other than thrombin have been demonstrated to activate factor VIII. Factor Xa is the most likely protease to augment the activity of thrombin. While factor Xa can partially activate factor VIII, it is clear that complete activation is accomplished only with thrombin (41, 42). It has been reported that factor IXa can activate Factor VIII but the contribution of this reaction to factor VIII cofactor function would be far less significant than that catalyzed by thrombin or factor Xa (43). In vitro studies would suggest that thrombin activation is requisite for biological function (25, 27, 44-50). It is emphasized that factor VIII, once activated to factor VIIIa, decays quite rapidly.

Influence of von Willebrand Factor on Factor VIII Activity

The presence of von Willebrand Factor (vWF) appears to influence the in vitro assay of factor VIII. Highly purified recombinant factor VIII preparations which do not contain vWF gave lower-than-expected potency values when assayed in vWF-depleted factor VIII-deficient plasmas (51, 52). As a result, dilution of highly-purified factor VIII samples in hemophilia A plasma prior to assay has been recommended (53-55). In support of this concept, it has been shown that plasma vWF is required for a normal circulating half-life for factor VIII (56) and that vWf-free recombinant factor VIII associates with vWF after infusion (57). In addition to this effect on the state of factor VIII in the circulation, vWF also directly stabilized factor VIII (58).

While the results with the use of vWF-antibody-immunodepleted factor VIII-deficient plasma may result in an unsatisfactory assay for high-purity concentrates, such substrate plasmas continued to be useful for factor VIII assay in plasma samples (59). Equivalence as a diluent has been reported for hemophilia A plasma and factor VIII-deficient plasma obtained by treatment with EDTA (55). Rothschild et al. (60) have reported that a plasma depleted by both a factor VIII antibody and a vWF antibody yielded equivalent results compared to hemophilia A plasma with intermediate and high purity plasma-derived concentrates.

Clear biochemical evidence for the importance of vWF in a factor VIII assay system has not been demonstrated. vWF does not appear to have an effect on the thrombin-activation of factor VIII with respect to the increase in biological activity but does serve as a cofactor for a non-rate limiting step in the overall activation process (61, 62). These studies were performed with porcine factor VIII and it is not clear that such results would be obtained with the human protein. However, if these results could be effectively translated to the human system, a role for vWF in the assay systems would be clear considering the essential role of thrombin in the factor VIII activation process. For example, Vukovich et al. (63) presented data supporting a role for vWF in a two-stage assay but not in a one-stage assay. Somewhat the opposite conclusion was reached by other investigators who reported that factor VIII with greatly reduced vWF content appeared to have higher activity in the two-stage assay than in the one-stage assay (64).

vWF does appear to inhibit the activation of factor VIII by factor Xa (65, 66). In other studies, vWF has been reported to decrease the stability of both thrombin-modified and factor Xa-modified factor VIII (67). The issue of the influence of vWF on the assay of factor VIII in high purity concentrates versus plasma factor VIII or low-purity concentrates is complicated by the marked differences in vWF quality in the high-purity factor VIII concentrates (68-70). Aronson and Chang showed that while a recombinant factor VIII (Kogenate®) and a highly purified factor VIII concentrate obtained by immunoaffinity chromatography with a factor VIII antibody (Hemofil® M) readily associated in vitro with von Willebrand factor to form a more-rapidly sedimenting complex on ultracentrifugal analysis (71), another highly-purified concentrate obtained by immunoaffinity chromatography with a vWF antibody (Monoclate®) did not show this association in vitro with von Willebrand factor. There was no difference in the sedimentation behavior of the three therapeutic preparations after infusion into a hemophilic patient. Differences between Monoclate® and Hemofil® M have been demonstrated in a chromogenic substrate assay system (14) and in data shown below. The molecular basis for the assay discrepancies is not clear but it is unlikely to be related to the activation state of factor VIII since factor VIIIa (activated factor VIII) does not effectively bind to von Willebrand factor (72).

In Vitro Assay Systems for Factor VIII

Three types of assay systems exist for the measurement of factor VIII in biological samples (73). These are the one-stage partial thromboplastin time using factor VIII-deficient plasma (11, 12), the two-stage assay (74, 75) and chromogenic substrate assays (76, 77).

Table 1 Comparison of two ultra-high purity factor concentrates with two chromogenic assays and the activated partial thromboplastin time

Sample[a]	APTT	Dade-Behring	Chromogenix	Label Potency
Concentrate A	21.6[b]	20.4	17.1	23.5
Concentrate B	98.5	86.2	50.2	100

a Samples had been previously dissolved in deionized water and stored at -70oC. The samples were prediluted in Hemophilia A plasma prior to assay.

b Values are presented as units of factor VIII:C activity/mL sample obtained with the Mega I standard

The one-stage assay for factor VIII measures the ability of a sample to shorten the clotting time of severe hemophilia A plasma after contact activation and recalcification. This assay uses the intrinsic system in which the reaction is initiated via contact activation with fibrinogen clotting as the endpoint. In the one-stage assay for factor VIII, factor VIII-deficient plasma is preincubated with phospholipid containing contact activation material such as kaolin or elagic acid and the sample; the assay itself is initiated by the addition of calcium chloride. The use of this assay assumes that factor VIII in the sample is rate-limiting with all other necessary components (clotting factors, phospholipid or activated platelets and other cofactors) present at saturating levels. The aPTT, while likely the assay system most closely related to physiologic function, has some major drawbacks. The assumption that all components other than factor VIII are present at saturating levels is not necessarily valid and the sample may contain components other than factor VIII which could contribute to or partially oppose the measured activity. An example of this is provided by the observations on the apparent influence of vWF on observed factor VIII activity (51, 52). The presence of trace amounts of factor VIII in the substrate plasma also can influence results (78). Finally, there is evidence that each factor VIII-deficient plasma will give a somewhat unique slope for the standard curve.

The two-stage assay system consists of two separate reaction periods. The first incubation contains the factor VIII sample in combination with a serum reagent which contains human serum (source of factor IXa and factor X), bovine serum absorbed with barium salts (source of factor V/Va), calcium ions and phospholipid. The prothrombinase (79) which is formed is measured in the second stage by the ability to activate prothrombin to thrombin with the rate of fibrinogen conversion to fibrin being measured. Since the two-stage assay is an isolated system, there is (a) no physiologically-significant thrombin feedback activation of Factor VIII and (b) no modulatory effect of plasma protease inhibitors (serpins).

The third type of assay system available is the chromogenic substrate assay(s). It should be noted that the chromogenic substrate assay systems are so-named not because of a similarity in reaction mechanism, but rather that a colored product, p-nitroaniline, is the signal measured (80). Chromogenic substrate assays are related to the two-stage assay in that there are usually two incubation periods. The first, which actually measures factor VIII cofactor activity, involves the reaction of factor IXa, calcium ions, factor X, phospholipid and the sample or reaction blank; factor Xa is produced during this incubation period. The second incubation period measures the factor Xa produced in the first incubation period, using a peptide nitroanilide substrate. The hydrolysis of the peptide nitroanilide substrate forms p-nitroaniline which is usually measured by absorbance at 405 nm.

Currently, there are four chromogenic substrate assay systems which are commercially available; these chromogenic substrate assays are not equal with respect to mechanism:

(1) The original Chromogenix system contains factor X, factor IXa, prothrombin, calcium ions, phospholipid and the factor VIII sample/standard/blank.

(2) The Dade-Behring, the new Chromogenix (81) and Biopool systems contain factor X, factor IXa, thrombin, calcium ions, phospholipid and the factor VIII sample/standard/blank. These three systems contain thrombin which is added to maximize factor VIII activation such that there would be improved molecular homogeneity of factor VIII during assay.

All of these assay systems consist of two stages. The first stage generates factor Xa, the difference between the two types of systems is that the Dade-Behring, the new Chromogenix and the Biopool systems contain thrombin while with the first Chromogenix assay thrombin must be generated during the first stage incubation. The second stage is essentially identical for the various systems in that there is a chromogenic factor Xa substrate, buffer and a thrombin inhibitor. It is the hydrolysis of the chromogenic substrate that is actually measured in the assay. The rate of hydrolysis will vary between methods depending on the chromogenic substrate used. Although all modern chromogenic substrates have reasonable specificity, some cross-reactivity cannot be discounted. All formulations on the market at present contain thrombin inhibitors preventing thrombin interference in the assay. A new chromogenic substrate assay has been reported by Immuno. The Immuno assay uses all human proteins but contains no thrombin (82).

What about comparison of assay results? As has been mentioned above, there have been continuing reports of discrepancies between assay systems. While not wishing to complicate the issue, Table 1 presents a comparison between two ultra-high purity factor VIII concentrates with three assay systems. The point of this comparison is not to argue for the superiority of one concentrate vs. another but rather to emphasize the importance of thrombin in the chromogenic assay system. It is clear that with either concentrate, the aPTT is the most accurate measure when compared to label potency; the Dade-Behring assay system which contains thrombin would appear to be more accurate that the original Chromogenix system.

Concentrates: Problems in the Assay of High Purity Factor VIII

The accurate determination of factor VIII potency in therapeutic concentrates is of critical importance to all participants: manufacturers, treaters and patients. In the final analysis it is the *in vivo* hemostatic effectiveness which is decisive in dosing considerations. What then is the most reliable *in vitro* assay to predict *in vivo* performance? Our thesis is that the one-stage activated partial thromboplastin time (aPTT) is at present the most accurate assay for the determination of factor VIII potency in therapeutic concentrates. This recommendation is based not on theoretical considerations but rather on the observed correlation with clinical performance with the potency of factor VIII preparations (83). However, recognizing that the chromogenic assays provide advantages in terms of more facile integration into laboratory information management systems and the lack of necessity of using hemophilia A plasma, we submit that a chromogenic assay system could be effectively used for potency assessment and patient management only if there is clinical experience to verify that vial potency designation is meaningful in the determination of dose and that this dosage translates by determination of patient plasma factor VIII activity into satisfactory control of bleeding under the full spectrum of clinical experience.

Factor VIII Assay Results: In Vivo Studies

Earlier studies with less highly-purified concentrates reported that two-stage activity was increased as compared to one-stage activity. When the two assays were used to follow the *in vivo* pharmacokinetics of factor VIII, the one-stage assay gave a yield equivalent to 100% recovery while a lower recovery (80%) was obtained with the two-stage assay (84). Similar results were obtained by other investigators with an intermediate purity concentrate (85). The one-stage assay gave $109 \pm 20\%$ of predicted activity recovery while the two-stage assay gave $92 \pm 14\%$. The Chromogenix Coatest assay gave a similar result ($91 \pm 11\%$). Smaller differences in *in vivo* recovery with intermediate concentrates using the three assay systems were observed by other investigators (86).

While the one-stage assay has been criticized as being unreliable (87), it appears to remain as the assay of choice for the management of patients with hemophilia in the clinic on a global basis (83, 88-100).

Factor VIII Assay Issues to Be Resolved

Specific issues regarding the assay of factor VIII are as follows and must be resolved or reconciled for the successful interpretation of all factor VIII assay results.

First, in any assay system (one-stage, two-stage, chromogenic), factor VIII activity is measured by the rate of factor X activation. Given our current understanding of factor VIII, if the factor VIII sample contains any species other than fully activated (i. e. thrombin-activated) factor VIII, the assay will then, in fact, be a coupled system. With unactivated factor VIII, conversion to factor VIIIa must be effectively accomplished before its participation as a cofactor in the activation of factor X; as such, without full activation of factor VIII, it is possible that the assay system would measure the rate of conversion of factor VIII to factor VIIIa rather than the amount of factor VIII present. This system is comparable to other coupled assay systems used in clinical chemistry (101-104); in this context, the generation of factor Xa by factor IXa is dependent upon the rate of generation of factor VIIIa such that the activation of factor X is coupled to the generation of factor VIIIa. Any coupled assay system must be rigorously validated to demonstrate unequivocally that the measured response is directly related to the concentration of the substance being measured. In other words, in the case of factor VIII, the amount of factor Xa formed in a specific time period and measured either by rate of formation (kinetic rate assay) or amount formed after a specific period of time (endpoint assay) must be proportional to the amount of Factor VIII in the system. While this may be accomplished in the chromogenic assays cited herein, it is somewhat more difficult for either the two-stage or one-stage assay systems. With respect to the nature of the factor VIII molecular species present, the activation step must not be rate limiting. Molecular homogeneity would be most easily accomplished in an isolated system (either two-stage or chromogenic) by fully activating the factor VIII sample prior to assay while assuring that there is sufficient factor IXa, calcium ions and phospholipid to stabilize the activated factor VIII during the assay.

Secondly, how does vWF affect factor VIII function and does the quality of the vWF matter? The quality issue refers to whether there is a difference between high-molecular weight vWF, intermediate molecular-weight vWF and vWF dimer. The potential differential effect of vWF (quantitative and qualitative) on the various assay systems must be elucidated for successful interpretation of results; in particular, the influence of the hemophilic plasma used for dilution of the high purity concentrates must be explained.

Third, there is the general issue of the *in vivo* recovery of ultra-high purity factor VIII concentrates with particular emphasis on the recombinant DNA products (Kogenate®, Recombinate® and Refacto® (105). There has been considerable recent interest in problems associated with Refacto®. Refacto® is a B-domainless recombinant factor VIII therapeutic developed by Pharmacia-Upjohn (106-108) which is being commercialized by Genetics Institute. While primary data is not available with the use of Refacto® in hemophilia A subjects, Lusher and colleagues (109) state that "While recovery values are higher with the chromogenic assay than with the one-stage assay for the two full-length recombinant FVIII preparations, a unique discrepancy exists for the B-domainless rFVIII preparation, Refacto®. With Refacto®, recoveries measured by the chromogenic assay are as expected, whereas one-stage methods give values of approximately 50% of that expected." Mikaelsson et al. (110) reported that there are marked differences in the phospholipid requirement in the assay of Refacto® compared to the other factor VIII preparations. This might explain the differences observed by Lusher et al. (109). In earlier studies on B-domainless factor VIII, Pittman et al. (111) note similar activity values for either the Coatest chromogenic or the one-stage assay. In a study of some structurally-related factor VIII derivatives, Mertens et al. (112) report large differences between the chromogenic assay (cofactor activity) and the one-stage assay with hemophilia A substrate plasma (procoagulant activity). This is a complex problem which will require study in larger patient populations.

Finally, regardless of the type of assay chemistry being used, in order to have valid assays one must adhere to appropriate statistical standards (c.f. 113). An appropriate dilution curve of both sample and standard must demonstrate linearity, a significant regression (i. e. concentration dependence) and the lines for standard and sample must be parallel.

Conclusions

Given the current status of information on the assay of factor VIII in therapeutic concentrates, we suggest the following recommendation pro tem.

1. The aPTT assay is an accurate and sensitive method for both the determination of factor VIII potency in therapeutic concentrates and for the subsequent measurement of factor VIII in hemophilic recipients.
2. Chromogenic Assays can also be accurate measures of factor VIII activity both in high-purity concentrates and patient samples if there is an appropriate thrombin activation step included in the assay process. Failure to include this step will result in an inaccurate measure of factor VIII activity in high-purity concentrates. Relative to this requirement is the need for the presence of sufficient factor IXa, calcium ions and phospholipid to stabilize factor VIIIa during the assay. An equally important requirement is the demonstration of the clinical relevance of the potency designation as established by the chromogenic assay system.

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